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Award Number: W81XWH-06-1-0697

TITLE: The Role of Protein Kinase D (PKD) Signaling in Breast Cancer Cell Migration and Invasion

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REPORT DATE: September 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE (DD-MM-YYYY) 01-09-2007		2. REPORT TYPE Annual Summary		3. DATES COVERED (From - To) 30 AUG 2006 - 29 AUG 2007	
4. TITLE AND SUBTITLE The Role of Protein Kinase D (PKD) Signaling in Breast Cancer Cell Migration and Invasion				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-06-1-0697	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Claudine Christoforides E-Mail: cvc@hms.harvard.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Beth Israel Deaconess Med. Center Boston, MA 02215				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The goal of my project as outlined in the original application is to analyze the role of Protein Kinase D (PKD) in breast cancer cell motility, the phenotype critical for metastasis. The work I have conducted includes the establishment of gene expression system in breast cancer cell lines vital for a comprehensive study of contribution of PKD towards invasive migration. By means of these systems, I have demonstrated that loss or inhibition of PKD results in a migration defect phenotype in a highly metastatic breast cancer cell line. I have also found that loss or inhibition of PKD results in apoptotic cell death.					
15. SUBJECT TERMS Protein Kinase D (PKD), breast cancer cell migration and invasion, retroviral overexpression, lentiviral silencing, apoptosis					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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Introduction

The goal of my project as outlined in the original application is to analyze the role of Protein Kinase D (PKD) in breast cancer cell motility, the phenotype critical for metastasis. PKD is a serine/threonine kinase that has been best characterized as a key regulator of vesicular fission in golgi trafficking (1). It has also been reported that PKD promotes survival in cells exposed to oxidative stress signals (2). Based in part on a recent study which links PKD to fibroblast cell migration (3), I hypothesize that a PKD and Rho GTPase signaling network controls cytoskeletal reorganization and cellular adhesion, thereby regulating cell motility. I am testing the invasive migration of both PKD-overexpressing, -knockdown, and -inhibited cells in transwell assays using 3T3-conditioned media as chemoattractant. I aim to determine the specific mechanism and PKD substrates that control this phenotype. Mutant PKD constructs refractory to silencing by shRNA will be introduced into PKD-knockdown cells to determine the contribution of the specific motifs and phosphorylation sites that I have mutated. To better understand the involvement of Rho GTPases in PKD-mediated invasive migration, reporter GTPase constructs will be introduced into both PKD-overexpressing and PKD-knockdown cells and analyzed for activity. Additionally, I will characterize the expression, activation state, and localization of PKD in breast cancer sections and cell lines from invasive tumors. This work will determine the regulation and function of a previously uncharacterized signaling pathway that is critical for breast cancer progression, the Rho-PKD signaling axis, and how it impacts invasive migration. The results of this research will yield an increased understanding of mechanisms that control the metastatic phenotype of breast carcinoma cells, subsequently allowing for new therapeutic strategies targeted to advanced stage tumors.

Body

The following tasks from the Statement of Work for this project were the focus for the research period from 30 September 2006-30 September 2007:

To determine whether PKD controls cytoskeletal reorganization and a migratory/invasive phenotype in epithelial cells (months 1-15).

- a. Optimize retroviral infection technique to induce over-expression of wild type and various mutant alleles of PKD in various breast carcinoma cell lines (months 1-2).
- b. Optimize lentiviral infection technique to silence expression of PKD in these cell lines (months 3-6).
- c. Analyze the invasive/migratory capacity of PKD-expressing and PKD-silenced cells using a matrigel coated transwell assay (months 7-9).
- d. Characterize actin stress fiber formation and focal adhesion assembly using immuno-fluorescence microscopy and immunohistochemistry (months 10-12).

Progress

- a. Optimize retroviral infection technique to induce over-expression of wild type and various mutant alleles of PKD in various breast carcinoma cell lines.

After I obtained the necessary reagents for retroviral infection, I was able to generate data using this system. However, within a few months, the system began to fail. The consequence of infection was cell death. I recently determined that the preparatory isolation of the DNA was yielding contaminated samples. I have resolved this issue and am currently subcloning a number of mutant PKD alleles that I created in a transient transfection vector into the retroviral expression vector. This will be a high-efficiency overexpression system that will allow me to probe the contribution of the specific motifs and phosphorylation sites in PKD that I have mutated towards invasive migration.

- b. Optimize lentiviral infection technique to silence expression of PKD in these cell lines.

In addition to initiating the use of this system in our lab, I have obtained two distinct short-hairpin PKD-silencing constructs in the PLKO lentiviral-infection vector. As this system is now widely used

in the Toker lab, we have cooperatively optimized it to an extremely high efficiency and with very little negative impact on cellular viability.

c. Analyze the invasive/migratory capacity of PKD-expressing and PKD-silenced cells using a matrigel coated transwell assay.

Over-expression studies involving both transient transfection and retroviral infection of a constitutively active mutant allele of PKD resulted in decreased cellular migration and invasion in MDA 231 breast cancer cells (which express low levels of PKD) in a transwell assay in response to chemoattractant. Knockdown of PKD1 by lentiviral silencing also resulted in decreased migration in HS578T breast cancer cells (which express moderately high levels of PKD). Since submitting the original proposal, an exciting new tool for probing the function of PKD has been made available to me. Our lab collaborates with CRT (Cancer Research Technology) towards the development of small molecule inhibitors of PKD. They have created a panel of PKD inhibitors with varying potencies and specificities that we are testing in our breast cancer cell lines. Use of a PKD-specific inhibitor in combination with PDBu (phorbol 12,13-di-butyrate, a potent activator of PKD) yielded a migration defect in HS578T breast cancer cells compared to cells treated only with PDBu. This phenotype, which results from the use of either PKD-knockdown or PKD-inhibited cells is consistent with the published finding that introduction of a kinase-inactive mutant allele of PKD impaired motility of a fibroblast cell (1). However, it was unexpected that both over-expression and knockdown of PKD would result in the same phenotype.

There are two likely explanations for this phenomenon:

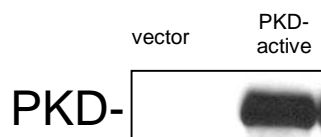
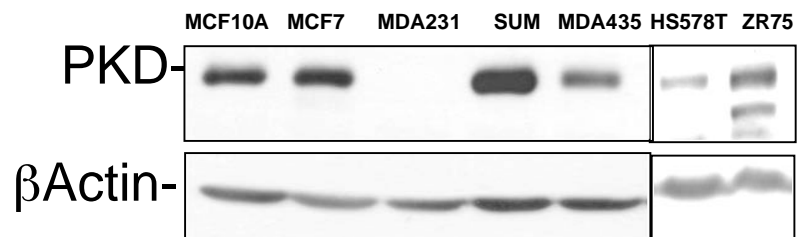
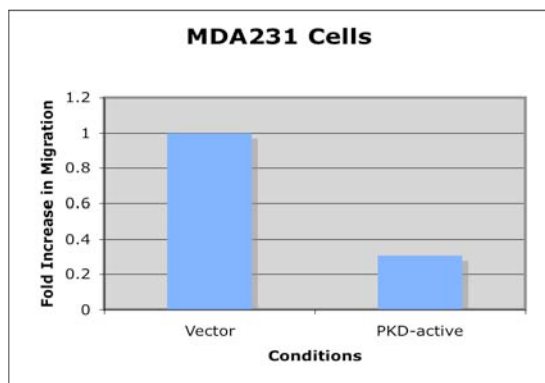
1) A specific physiological level of PKD is required for a coordination of spatiotemporal signals that control cell migration.

2) PKD activity is required for cell adhesion; following either over-expression of knock-down of PKD, cells which are either too adherent or not adherent enough become less migratory.

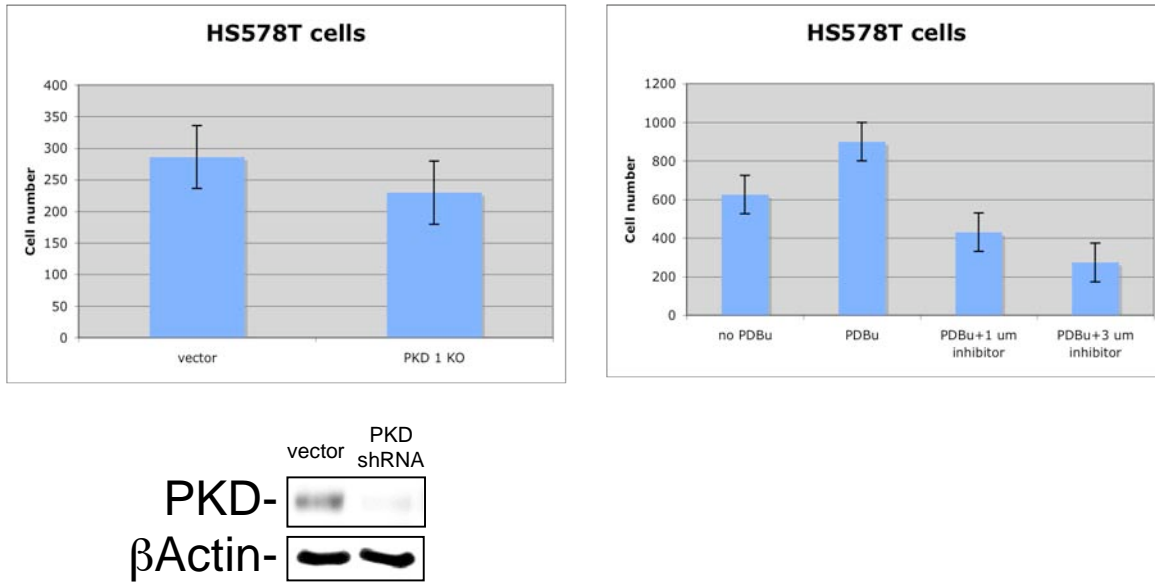
Breast cancer cell lines, PKD knockdown/migration phenotype

Cell Line	Origin	Tumorigenicity	PTEN Status	P53 Status	PKD expression	PKD knockdown/migration
ZR75	Infiltrating ductal carcinoma, ascites	+	+	wild-type	moderate	Significantly increased (not yet repeated)
MCF7	Breast adenocarcinoma, pleural effusion	+	+	wild-type	high	Not migratory enough to evaluate effect
MCF10A	Basal/myoepithelial derived	-	-	wild-type	high	Not migratory enough to evaluate effect
MDA231	Breast adenocarcinoma, pleural effusion	+ (metastatic)	-	mutant	low	Overexpression → impaired
SUMPT159	Breast adenocarcinoma, primary tumor	+ (metastatic)	-	mutant	high	TBD
MDA435	Metastatic ductal adenocarcinoma	+ (metastatic)	-	mutant	high	TBD
Hs578T	Ductal carcinoma, primary tumor	+ (metastatic)	-	mutant	moderate	Significantly impaired

Overexpression of PKD results in a migration defect in MDA231 breast cancer cells



PKD knockdown and inhibition impair cellular migration in a highly metastatic breast cancer cell line



Unexpectedly, knockdown of PKD resulted in increased migration of ZR75 cells.

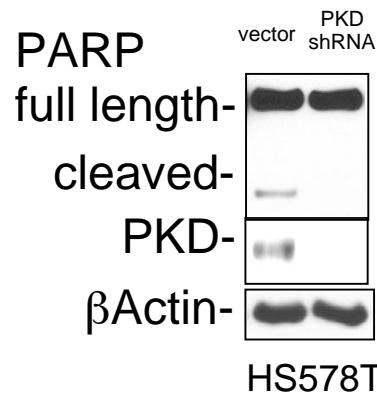
However, I have only conducted this experiment once and must repeat it to determine if this finding is reproducible. I have determined that both MCF7 and MCF10A cell lines are not migratory enough to evaluate the contribution of PKD towards invasive migration. I am currently determining the migration and invasion phenotypes of PKD knockdown in other breast cancer cell lines.

To confirm that these findings are due specifically to the loss of PKD, I created mutants that are refractory to shRNA silencing. These constructs are designed to be transiently transfected into PKD-knockdown cells to analyze their ability to rescue the migration defect. However, I have had significant difficulty achieving a high enough transfection efficiency to determine their contribution to migration in the transwell assay. I intend to both optimize the transfection procedure and subclone the cDNAs into a retroviral expression vector, which guarantees a high efficiency of expression.

An unanticipated but reproducible observation I have made is that PKD knockdown cells have significantly decreased viability. The monolayer of PKD-knockdown cells on an untreated

cell culture plate are, after 24 hours, significantly less confluent than control cells. This difference in confluence increases over time. One explanation for this finding is the induction of caspase-mediated apoptosis subsequent to loss of PKD. To investigate this possibility, lysates from both PKD knockdown and inhibitor treated cells were probed using an antibody for PARP (Poly (ADP-ribose) Polymerase), the cleavage of which is a reporter for the apoptotic event. Noticeable PARP cleavage occurred in these cells while no cleavage was apparent in control cells. Specific inhibition of PKD in adherent cells results in apoptotic vacuoles after an overnight treatment. This observation that knockdown or inhibition of PKD results in impaired cell viability is consistent with the report that PKD protects cells from death in response to genotoxic reagents (2). While the partial knockdown of PKD in this report sensitized cells to chemically induced death, a more complete knockdown of PKD alone initiates death. This difference may be explained by the high efficiency of knockdown using lentiviral silencing system as opposed to the transient transfection of siRNA.

**PKD Inhibitor causes apoptosis in
HS578T breast cancer cells**



d. Characterize actin stress fiber formation and focal adhesion assembly using immuno-fluorescence microscopy and immunohistochemistry.

The observation that PKD loss of function results in cell death, which likely causes defective migration has impacted the direction of my thesis studies. This exciting, unanticipated finding could reveal an unexpected role for PKD in cell viability and elucidate the mechanism by which PKD controls cell motility. I have therefore chosen to investigate the validity and potential mechanisms of this phenomenon before fulfilling this sub-task.

Current research and future directions

A primary ongoing effort is to investigate the contribution of PKD towards cellular migration and invasion in a number of breast cancer cell lines using both retroviral overexpression and lentiviral silencing. I will use a panel of PKD alleles refractory to silencing by shRNA and with mutations in specific motifs and phosphorylation sites to analyze their contribution to both cancer cell migration and invasion. To investigate the role of PKD in cell viability, I plan to determine the timeframe and mechanism of death. I will quantitatively assess viability using TUNEL staining and a colorimetric proliferation assay such as MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] quantification on the PKD knockdown and inhibitor treated cells. I will determine whether this phenotype can be blocked using a caspase inhibitor.

To determine the involvement of Rho GTPases and potential PKD substrates in cellular viability and motility, I will analyze the activity of these proteins in response to both the presence and absence of activated PKD. I will test whether the migration defect and impaired

viability of PKD-knockdown cells can be rescued by the introduction of activated alleles potentially involved in the PKD-signaling pathway.

Additionally, I plan to visualize PKD-overexpressing, -knockdown, and -inhibited cells using live cell microscopy to determine both the localization of PKD and the specific morphological defects that account for the viability and migration phenotypes.

Key Research Accomplishments and Reportable Outcomes

- Optimization of both overexpression and silencing cell infection systems with which to conduct gain and loss of function experiments using breast cancer cell lines in invasive migration transwell assays.
- In collaboration with CRT, the development and testing of a panel of PKD-specific inhibitors.
- Determination of a reproducible migration-defect phenotype of PKD-knockdown and inhibition in HS578T breast cancer cells, which are highly metastatic and of PKD-overexpression in MDA231 cells.
- Preliminary observations of a previously uncharacterized effect of PKD loss on cellular viability.

Conclusion

The work I have conducted on Task 1 of the Statement of Work includes the establishment of systems vital for a comprehensive study of the role of PKD in breast cancer cell invasive migration. By means of these systems, I have demonstrated that loss or inhibition of PKD results in a migration defect phenotype in a highly metastatic breast cancer cell line. I have found that PKD-knockdown results in PARP cleavage, which is a feature of apoptosis. Consistent with this observation, inhibition of PKD decreases cellular viability and causes the appearance of apoptotic vacuoles. A thorough investigation of the specific cause of impaired viability could explain the migration defect and potentially uncover a role in breast cancer tumorigenesis.

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